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ELECTROPHORETICALLY MONODISPERSE CYTOCHROME C OXIDASES

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Summary: A discontinuous gradient polyacrylamide gel electrophoresis under nondenaturing conditions has been used to demonstrate monodispersity of procaryotic and eucaryotic cytochrome c oxidase preparations. Alkaline treated bovine enzyme which contains nine subunits as analysed by subsequent discontinuous SDS-polyacrylamide gel electrophoresis is a monodisperse dimer in 0.1% Triton X-100 and a monomer in 0.1% dodecyl maltoside. The Mr-values corrected for bound detergent are 286,000 in Triton X-100 and 152,000 in dodecyl maltoside respectively. The two-subunit bacterial cytochrome c oxidase of Paracoccus denitrificans is proved to be a monomer with a corrected Mr of 76,000 in both nonionic detergents Triton X-100 and dodecyl maltoside.

Cytochrome c oxidase is the terminal enzyme of the respiratory electron-transport chain. It is integrated into the plasma membrane in procaryotes and into the inner mitochondrial membrane of eucaryotes respectively. The complexity of this intrinsic membrane protein is quite different depending on its origin. The bacterial enzyme of Paracoccus denitrificans consists of two (1) or three (2) different subunits only whereas the so far most complex oxidase isolated from bovine heart mitochondria contains 13 subunits in stoichiometric amounts (3,4).

Problems in physicochemical experiments and in crystallization may be caused by the polydispersity of cytochrome c oxidase which partially is a consequence of the low stability of the solubilized complex. Different quarternary structures of beef heart cytochrome c oxidase due to subunit heterogeneity could be identified by anion-exchange FPLC (5) and by native DGPAGE (6). The enzyme disintegrates by loss of subunits III, VII, VIII, II in small

Abbreviations: FPLC, fast protein liquid chromatography; native DGPAGE, discontinuous gradient polyacrylamide gel electrophoresis under nondenaturing conditions; SDS-DPAGE, discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

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amounts and possibly X as shown by FPLC and subsequent SDS-DPAGE (5) or subunits III, VI, VII, VIII, X and particularly IX as determined by 2D-PAGE analysis respectively (6). Cytochrome oxidase subunits are designated according to our recently introduced nomenclature based on the polypeptide molecular mass data (7).

In the present study we report that monodisperse complexes of beef heart cytochrome c oxidase are obtained following alkaline treatment by which most of the subunits causing heterogeneity are removed. Alkaline treated bovine enzyme is compared with conventionally isolated enzyme by two-dimensional polyacrylamide gel electrophoresis. A nondenaturing discontinuous gradient separation system (native DGPAGE) supplemented by nonionic detergent either Triton X-100 or dodecyl maltoside in the 1st dimension is combined with a discontinuous nongradient SDS-PAGE system in the 2nd dimension (6). Cytochrome c oxidase from Paracoccus denitrificans which misses the homologous subunits causing heterogeneity therefore is expected to be monodisperse. By native DGPAGE the bacterial oxidase is shown to be a monomer built up by two subunits as analyzed by subsequent SDS-DPAGE.

MATERIALS AND METHODS

Isolation of cytochrome c oxidases: Bacterial enzyme was isolated from Paracoccus denitrificans according to (8). After the final preparation step carried out by FPLC equipped with the anion-exchange column Mono-Q (Pharmacia HR 10/10), Paracoccus oxidase was precipitated in ammonium sulfate and redissolved in standard buffer (63 mM Tris-phosphate, pH 7.3, 0.1% nonionic detergent either Triton X-100 or dodecyl-ß-D-maltoside). Small aliquots were stored at -80 °C. Beef heart cytochrome c oxidase was isolated essentially as in (9) with ommission of the last dialysis step. The centrifugation pellet of a final ammonium sulfate precipitation in 1% cholate was dissolved in standard buffer. This preparation was stored at -80 °C and used for alkaline treatment.

Alkaline incubation of beef heart cytochrome c oxidase (1mg/ml) was performed by dialysis against 0.375 M Tris / 0.075 M glycinate buffer, pH 9.5 (25 °C), containing 0.1% nonionic detergent, either Triton X-100 or dodecyl maltoside, for 1 h at 4 °C. The solution was neutralized by subsequent dialysis against standard buffer and the precipitate which formed at this step was removed by centrifugation. Then the enzyme was precipitated by addition of ammonium sulfate. After centrifugation of the turbid solution, the oxidase was redissolved in standard buffer. The total procedure was repeated over three cycles. A final anion-exchange chromatography on a DEAE-Sepharose column (Pharmacia SR 10/50) was performed as previously described (6).

Two-dimensional polyacrylamide gel electrophoresis: Native DGPAGE in the 1st dimension was carried out according to (6), stacking on a 3.13% gel at pH 7.3, separation on a 4/30 gradient slab gel at pH 8.6. Prior to native DGPAGE both bacterial and mammalian enzyme were dialyzed 24 h into standard buffer to which glycerol to a final concentration of 10% was added. The 2nd dimension SDS-DPAGE system according to (10) was applied as previously described (6), just as further procedures: fixing, amido black staining, destaining and laser densitometry. Additional silver staining was performed essentially as in (11).

Binding of detergent to cytochrome c oxidase within the gel matrix was calculated from independent quantitative determination of both detergent by liquid szintillation counting and protein by colorimetry respectively. The native DGPAGE system as well as the last dialysis system prior to electrophoresis were supplemented with radioactive detergent in a 10-fold molar excess over oxidase (^{3}H-labeled Triton X-100, Roehm & Haas, 0.25 mCi/g; ^{3}H-labeled dodecyl-ß-D-maltoside, Amersham, purified according to (12), diluted to a final radioactivity of 0.50 mCi/g). Immediately after native DGPAGE two series of gel pieces different in area either containing protein or not were excised from the gel and crushed up. The radioactivity was measured and plotted versus gel volume. From the vertical distance between the two parallels obtained the amount of bound detergent to cytochrome c oxidase was calculated after the protein content was determined by the bicinchoninic acid method (13).

RESULTS AND DISCUSSION

Mammalian enzyme: After alkaline treatment of bovine heart oxidase and subsequent anion exchange chromatography when the polydispersity of the enzyme is reduced (14), more than 95% of the total protein content are found to be in one band of native DGPAGE gels (cf. fig.1). As the 2-D pattern in fig.2 shows and as further listed in the Table depletion of subunit III by alkaline treatment is accompanied by the loss of cytoplasmic polypeptides as previously reported (5,6,18,19), here VII, VIII and X. The apparent M_r-values all of which were determined at pore limit conditions of the native DGPAGE, for alkaline treated oxidase amount to 480,000 in Triton X-100 and to 310,000 in dodecyl maltoside respectively. Linear M_r-calibration curves could be established using the proteins thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin. The ratios of bound nonionic detergent within the native DGPAGE gels estimated

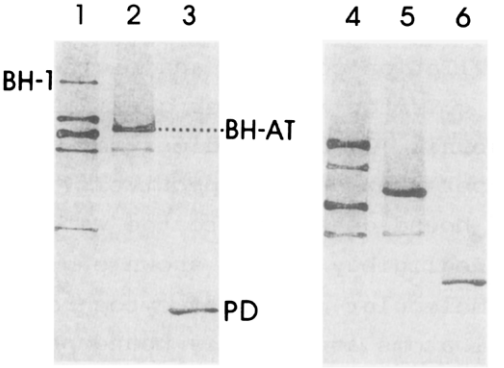

Figure 1. Discontinuous gradient polyacrylamide gel electrophoresis of cytochrome c oxidase under nondenaturing conditions (native DGPAGE). Separation was performed as described in (6) in the presence of 0.1% nonionic detergent either Triton X-100 (lanes 1-3) or dodecyl-ß-D-maltoside respectively (lanes 4-6). Lanes 1 and 4, 30 µg freshly prepared beef heart enzyme, lanes 2 and 5, 18 µg alkaline treated beef heart enzyme after anion-exchange chromatography, lanes 3 and 6, 12 µg cytochrome oxidase from Paracoccus denitrificans were applied.

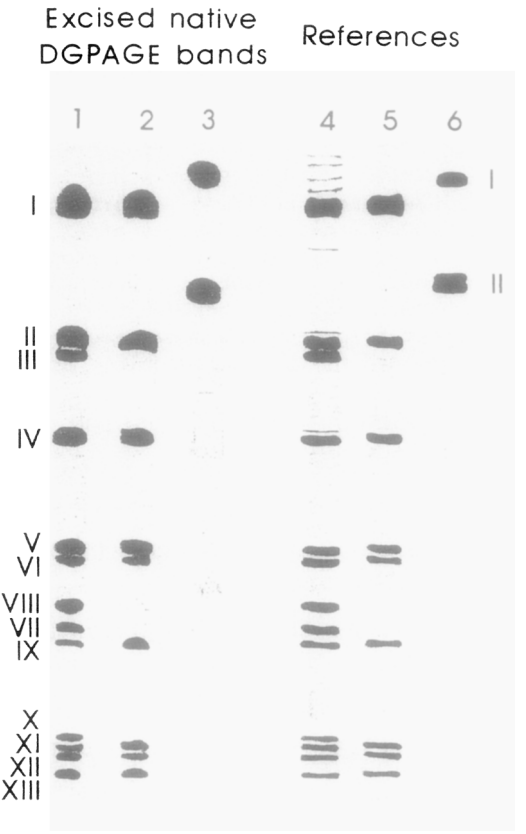

Figure 2. Two-dimensional polyacrylamide gel electrophoresis of cytochrome c oxidase. From the 1st dimension native DGPAGE gel supplemented by Triton X-100 (cf.fig.1, lanes 1-3) bands named below were excised and denatured; then polypeptides were resolved by 2nd dimension SDS-DPAGE: lane 1, band 1 of freshly prepared beef heart enzyme (BH-1), lane 2, major band of alkaline treated beef heart enzyme (BH-AT), lane 3, single band of Paracoccus dentrificans enzyme (PD) were applied. References: lane 4, freshly prepared, lane 5, alkaline treated bovine cytochrome oxidase, either containing 5 µg of protein, lane 6, 2 µg of freshly prepared bacterial enzyme were applied.

from liquid szintillation counting and bicinchoninic acid assay are (0.68 ± 0.05) g Triton X-100 per g oxidase in accordance to (14) for the subunit III depleted enzyme and (1.04 ± 0.08) g dodecyl maltoside per g oxidase respectively. Taking into account that the amount of bound detergent to the water soluble calibration proteins is negligibly low as shown e.g. for bovine serum albumin (20), the molecular masses of cytochrome c oxidase including heme-A, metal atoms and tightly bound phospholipids can be calculated from the apparent M_r-values and the binding of detergent. The corrected molecular masses are 286,000 in Triton X-100 and 152,000 in dodecyl maltoside representing a dimer and monomer respectively. For comparison the molecular mass of the alkaline treated monomer (147,144) is calculated from the protein chemical

TABLE

Polypeptide composition of beef heart cytochrome c oxidase complexes on native DGPAGE gels

Nomenclatures				Mr	N-terminal sequences	Band 1 of freshly prepared enzyme	Alkaline treated complex
Buse (7)	Buse (15)	Capaldi (16)	Kadenbach (10)				
I	I	I	I	56993	F-MFIN	+	+
II	II	II	II	26049	F-MAYP	+	+
III	III	III	III	29918	MTHQ	+	Ø
IV	IV	IV	IV	17153	AHGS	+	+
V	V	V	Va	12436	SHGS	+	+
VI	VIa	a	Vb	10668	ASGG	+	+
VII	VII	c	VIb	10063	Ac-AEDI	+	Ø
VIII	VIb	b	VIa	9419	ASAA	+	Ø
IX	VIc	VI	VIc	8480	STAL	+	+
X	VIIIc	VII's	VIIa	6243	FENR	+	Ø
XI			VIIb	≈6000	IHQK	+	+
XII	VIIIa	VII's	VIIc	5541	SHYE	+	+
XIII	VIIIb	VII's	VIII	4962	ITAK	+	+

Note. Our novel nomenclature for the subunits of cytochrome c oxidase (7) is based on the available protein chemical data. The subunits are basically listed according to their molecular masses except for subunits II and III which keep their original Roman numerals on historical grounds. The N-terminal sequence of subunit XI is taken from (17).
Symbols: +, subunit present; Ø, subunit not present in the native DGPAGE band.

molecular mass of the 13 subunit complex (202,787, ref.3) and the molecular masses of the missing subunits III, VII, VIII and X.

Band 1 of the freshly prepared beef heart enzyme on native DGPAGE gels (cf. fig. 1) which represents the intact 13 subunit enzyme (cf. fig. 2) has an apparent M_r of 670,000 in Triton X-100 and 430,000 in dodecyl maltoside respectively. Binding of detergent was determined to be (0.62 ± 0.06) g Triton X-100 per g oxidase and (0.94 ± 0.10) g dodecyl maltoside per g oxidase respectively both of which are in the same range as previously measured by gel filtration (21,22). The molecular masses of the 13 subunit cytochrome c oxidase corrected for bound detergent but including heme-A, metal atoms and phospholipids amount to 414,000 in Triton X-100 and to 222,000 in dodecyl maltoside respectively. Herewith previous results are confirmed indicating the existence of dimers under physiological conditions in the presence of Triton X-100 (14,18,21). The predominant aggregation state in the presence of dodecyl maltoside seems to be a monomer (23,24).

Bacterial enzyme: As shown in fig. 1 cytochrome c oxidase from Paracoccus denitrificans is monodisperse with an apparent M_r-value of 130,000 in Triton X-100 and 160,000 in dodecyl maltoside respectively. Its 2-D pattern is identical to the control on the SDS-DPAGE gel (cf. fig. 2) which confirms earlier results (25)

revealing the presence of two polypeptides with apparent M_r's of 45 kDa and 28 kDa. Bound detergent deducted, (0.71 ± 0.09) g Triton X-100 or (1.10 ± 0.16) g dodecyl maltoside per g oxidase, the corrected molecular mass of the two-subunit enzyme amounts to 76,000 in either nonionic detergent. In accordance the molecular masses of monomers estimated by gel filtration and ultracentrifugation are in the same range in the presence of dodecyl maltoside (19) just as in octyltetra/pentaoxyethylene (1).

According to the requirement of monodispersity, crystallization of cytochrome c oxidase preferably should be attempted using either procaryotic enzyme or alkaline treated mammalian enzyme after the stoichiometry of polypeptides and metals has been investigated by further studies.

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REFERENCES

(1) Ludwig, B., Grabo, M., Gregor, I., Lustig, A., Regenass, M. and Rosenbusch, J.P. (1982) J. Biol. Chem. 257, 5576-5578.
(2) Saraste, M., Raitio, M., Jalli, T. and Perämaa, A. (1986) FEBS Lett. 206, 154-156.
(3) Buse, G., Meinecke, L. and Bruch, B. (1985) J. Inorg. Biochem. 23, 149-153.
(4) Buse, G., Steffens, G.C.M., Biewald, R., Bruch, B. and Hensel, S. (1987) in: Cytochrome Systems, Molecular Biology and Bioenergetics, ed. S. Papa, Plenum Press, 261-270, in press.
(5) Finel, M. and Wikström, M. (1986) Biochim. Biophys. Acta 851, 99-108.
(6) Heinrichs, M. and Schönert, H. (1987) FEBS Lett. 223, 255-261.
(7) Buse, G., Steffens, G.C.M., Biewald, R., Bruch, B. and Hensel, B. (1987) in: Cytochrome Systems, Molecular Biology and Bioenergetics, ed. S. Papa, Plenum Press, 791-793, in press.
(8) Steffens, G.C.M., Biewald, R. and Buse, G. (1987) Eur. J. Biochem. 164, 295-300.
(9) Steffens, G.J. and Buse, G. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1125-1137.
(10) Kadenbach, B., Jarausch, J., Hartmann, R. and Merle, P. (1983) Anal. Biochem. 129, 517-521.
(11) Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197-203.
(12) Nalecz, K.A., Bolli, R. and Azzi, A. (1986) Methods in Enzymology 126, 45-64.
(13) Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76-85.
(14) Saraste, M., Penttilä, T. and Wikström, M. (1981) Eur. J. Biochem. 115, 261-268.

(15) Buse, G., Steffens, G.C.M., Meinecke, L., Biewald, R. and Erdweg, M. (1982) Eur. Bioenerg. Conf. Rep. 2, 163-164.
(16) Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Biochim. Biophys. Acta 726, 135-148.
(17) Takamiya, S., Lindorfer, M.A. and Capaldi, R.A. (1987) FEBS Lett. 218, 277-282.
(18) Georgevich, G., Darley-Usmar, V.M., Malatesta, F. and Capaldi, R.A. (1983) Biochemistry 22, 1317-1322.
(19) Nalecz, K.A., Bolli, R., Ludwig, B. Azzi, A. (1985) Biochim. Biophys. Acta 808, 259-272.
(20) Makino, S., Reynolds, J.A. and Tanford, C. (1973) J. Biol. Chem. 248, 4926-4932.
(21) Robinson, N.C. and Capaldi, R.A. (1977) Biochemistry 16, 375-381.
(22) Bolli, R., Nalecz, K.A. and Azzi, A. (1985) Arch. Biochem. Biophys. 240, 102-116.
(23) Nalecz, K.A., Bolli, R. and Azzi, A. (1983) Biochem. Biophys. Res. Commun. 114, 822-828.
(24) Suarez, M.D., Revzin, A., Swaisgood, M., Thompson, D.A. and Ferguson-Miller, S. (1983) Ann. Meet. Am. Soc. Biol. Chemists (Am. Soc. Biol. Chem. and Biophys. Soc. eds) Abstr. No. 1782, 2062.
(25) Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196-200.